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RESEARCH ARTICLE

A comparative study of peroxisomal structures in *Hansenula polymorpha pex* mutants

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Abstract

In a recent study, we performed a systematic genome analysis for the conservation of genes involved in peroxisome biogenesis (*PEX* genes) in various fungi. We have now performed a systematic study of the morphology of peroxisome remnants ('ghosts') in *Hansenula polymorpha pex* mutants (*pex1–pex20*) and the level of peroxins and matrix proteins in these strains. To this end, all available *H. polymorpha pex* strains were grown under identical cultivation conditions in glucose-limited chemostat cultures and analyzed in detail. The *H. polymorpha pex* mutants could be categorized into four distinct groups, namely *pex* mutants containing: (1) virtually normal peroxisomal structures (*pex7*, *pex17*, *pex20*); (2) small peroxisomal membrane structures with a distinct lumen (*pex2*, *pex4*, *pex5*, *pex10*, *pex12*, *pex14*); (3) multilayered membrane structures lacking apparent matrix protein content (*pex1*, *pex6*, *pex8*, *pex13*); and (4) no peroxisomal structures (*pex3*, *pex19*).

Introduction

In recent years, much progress has been made in identifying and characterizing proteins involved in peroxisome biogenesis. These proteins are called peroxins, and are encoded by *PEX* genes (Distel *et al.*, 1996). Up to now, 32 peroxins have been identified (Kiel *et al.*, 2006). With the exception of *PEX11*, the first 22 described *PEX* genes (*PEX1–PEX22*) all function in matrix protein import or in the formation of peroxisomal membranes. Yeast mutants in which a *PEX* gene has been deleted that plays a role in peroxisomal matrix protein import still maintain peroxisomal membrane structures (also termed peroxisomal ghosts) to which peroxisomal membrane proteins are normally sorted, whereas the bulk of the matrix proteins are mislocalized to the cytosol. Mutants defective in membrane formation (*PEX3* and *PEX19*) lack peroxisomal membranes and mislocalize both peroxisomal membrane and matrix proteins.

The most recently identified *PEX* genes, *PEX23–PEX32*, together with *PEX11*, are involved in the regulation of peroxisome abundance, size and distribution. Deletion of one of these genes generally does not have major effects on normal sorting of peroxisomal membrane and matrix proteins (Yan *et al.*, 2005).

The morphologic phenotype of yeast *PEX* deletion cells (*pex*) may provide important clues on the function of the deleted gene. For careful comparison of the effects of deletion of a given *PEX* gene, it is essential to analyze cells that have been grown identically and are in an optimal physiologic state. Hence, the use of batch cultures and peroxisome-inducing cultivation media (oleic acid, methanol) may have major disadvantages, as the *pex* mutant cells are generally unable to grow on these compounds. This may at least in part explain the wide range of peroxisomal phenotypes that have been described and that have often seemed contradictory (Hohfeld *et al.*, 1991; Baerends *et al.*, 1996; Wiemer *et al.*, 1996).

In this study, we analyzed *PEX1–PEX20* deletion mutants of the methylotrophic yeast *Hansenula polymorpha*, which are affected in peroxisome biogenesis and growth on methanol. To ensure reproducible, optimal cultivation and maximal peroxisome induction, *pex* mutant cells were grown in glucose-limited chemostat cultures, using choline as sole nitrogen source (van der Klei *et al.*, 1991). Oxidation of choline results in the release of formaldehyde, which subsequently induces the major peroxisomal enzymes involved in methanol metabolism, alcohol oxidase (AO), dihydroxyacetone synthase (DHAS), and catalase (CAT). The use of

steady-state chemostat cells has the advantage of highly controlled growth conditions (e.g. constant growth rate, pH, and temperature), allowing accurate comparisons between different strains. The data revealed that four distinct mutant peroxisomal phenotypes could be discriminated among the available *H. polymorpha pex* mutants.

Materials and methods

Organisms and growth

The *H. polymorpha* strains used in this study are listed in Table 1. The cells were grown in glucose-limited chemostat cultures at 37 °C in mineral medium (van Dijken *et al.*, 1976) containing 0.25% glucose as sole carbon source and 0.2% choline as nitrogen source. The dilution rate (*D*) was set at 0.1 h^{-1}. When needed, leucine was added to a final concentration of 30 μg mL^{-1}.

Escherichia coli strain DH5α was cultivated as previously described (Sambrook *et al.*, 1989).

Molecular techniques

Standard recombinant DNA techniques (Sambrook *et al.*, 1989) and genetic manipulations of *H. polymorpha* (Faber *et al.*, 1994) were performed as detailed previously. Restriction and DNA-modifying enzymes were obtained from Roche Molecular Biochemicals (Almere, the Netherlands). PCRs were performed with Expand High Fidelity PCR enzyme (Roche Molecular Biochemicals, Almere, the Netherlands) as described by the supplier, using a Perkin-Elmer GeneAmp PCR 2400 thermocycler. Oligonucleotides were synthesized by Baseclear (Leiden, the Netherlands) using a LiCore automated DNA sequencer and dye primer chemistry (LiCore, Lincoln, NE). For DNA and amino acid sequence analysis, the CLONE MANAGER 5 program (Scientific and Educational software, Durham) was used. The TBLASTN algorithm (Altschul *et al.*, 1997) was used to screen databases at the National Centre for Biotechnology Information (Bethesda, MD). Protein sequences were aligned using the CLUSTALX program (Thompson *et al.*, 1997).

Southern blot analysis was performed using the ECL direct nucleic acid labeling and detection system, as described by the manufacturer (GE Healthcare).

Isolation of the *H. polymorpha PEX2* gene

The *H. polymorpha PEX2* gene was isolated from the *H. polymorpha* genomic library pYT3 (Tan *et al.*, 1995) using degenerate primers (reverse primer, 5′-CACACG TAACARTANAYRTGNCCRCA-3′; forward primer, 5′-GAGTWCCAGAACAGRCARYTBSTNTGG-3′). Transformants were selected that carried vector pYT3 with an 8-kb insert. By subcloning, a 3-kb SalI–NdeI (blunt-ended) fragment was isolated. This fragment was ligated between SalI and SmaI into pBluescript II KS^+ (Stratagene, San Diego, CA) and sequenced. The nucleotide sequence of the *PEX2* gene was deposited at GenBank and assigned accession number AY688949.

Deletion of *PEX2*

For the deletion of *PEX2*, a plasmid with a 3-kb subclone containing *PEX2* was amplified by PCR using primer Del*PEX2*-1 (5′-AGATGATCACTAATGATTAGGCTATAG-3′), which introduces a BclI site upstream of the ATG start codon, and primer Del*PEX2*-2 (5′-AGAATCGATGGTCTATAATT AAACAAAA TATGCTGGCACGAAG-3′), which introduces

Table 1. *Hansenula polymorpha* strains used in this study

Strain	Relevant properties	Source or reference
Wild-type *leu1.1 ura3*	NCYC495 *leu1.1 ura3*	Gleeson & Sudbery (1988)
Wild-type *leu1.1*	NCYC495 *leu1.1 URA3*	Gleeson & Sudbery (1988)
pex1 leu1.1	*PEX1* disruption strain, *leu1.1*	Kiel *et al*. (1999)
pex2 leu1.1	*pex2* disruption strain, *leu1.1*	This study
pex3 leu1.1	*pex3* disruption strain, *leu1.1*	Baerends *et al*. (1996)
pex4 leu1.1	*pex4* disruption strain, *leu1.1*	van der Klei *et al*. (1998)
pex5 leu1.1	*pex5* disruption strain, *leu1.1*	van der Klei *et al*. (1995)
pex5 ura3	*Pex5* disruption strain, *ura3*	van der Klei *et al*. (1995)
pex6 leu1.1	*pex6* disruption strain, *leu1.1*	Kiel *et al*. (1999)
pex7 leu1.1	*pex7* disruption strain, *leu1.1*	This study
pex8 leu1.1	*pex8* disruption strain, *leu1.1*	Haan *et al*. (2002)
pex10	*pex10* disruption strain	Tan *et al*. (1995)
pex12 leu 1.1	*pex12* disruption strain, *leu1.1*	This study
pex13 leu 1.1	*pex13* disruption strain, *leu1.1*	Komori, Osaka, Japan, laboratory collection
pex14 leu 1.1	*pex14* disruption strain, *leu1.1*	Komori *et al*. (1997)
pex17 leu 1.1	*pex17* disruption strain, *leu1.1*	Komori, Osaka, Japan, laboratory collection
pex20 leu 1.1	*pex20* disruption strain, *leu1.1*	Otzen *et al*. (2005)

a ClaI site downstream of the stop codon. The resulting 4.7-kb PCR product was digested with NotI and Asp718I. The 1.3-kb NotI-blunt fragment and the 0.5-kb Asp7181I-blunt fragment were, in two subsequent steps, ligated into pBluescipt KS^+, using NotI–SmaI in the first step, and Asp718I–EcoRV in the second step. The plasmid was cut with BclI and ClaI and ligated with the *H. polymorpha URA3* gene (Merckelbach *et al.*, 1993), which was isolated as a BamHI–ClaI fragment, resulting in plasmid pHp*pex2*. The 4.1-kb deletion fragment was isolated from pHp*pex2* by digestion with BamHI and partial digestion with SalI, and used to transform *H. polymorpha* NCYC495 *leu1.1 ura3*. Methanol utilization-defective strains (Mut^-) were selected, and correct integration was confirmed by Southern blot analysis.

Isolation of the *PEX7* gene and construction of the deletion strain

The *H. polymorpha PEX7* gene was isolated from the *H. polymorpha* genomic library pYT3 using degenerate primers [5′ primer, 5′-AACTACGG(A/C)(C/T)TGGTNGG NAA(T/C)GG-3′; and 3′ primer, 5′-CCACA(C/G)TTT (G/A)A(T/C)(A/C)GANCC(G/A)TCCCA)-3′] based on conserved regions in *PEX7* genes of *Pichia pastoris*, *Saccharomyces cerevisiae*, *Homo sapiens* and *Kluyveromyces lactis*.

Transformants were selected that carried vector pYT3 with a 7-kb insert. By subcloning, a 2.7-kb XbaI–NruI fragment was isolated. This fragment was cloned into the SmaI site of pUC19, and analyzed by sequencing. The nucleotide sequence of *H. polymorpha PEX7* was deposited at GenBank and assigned accession number DQ217754.

A disruption mutant was created by cloning a 1.8-kb BamHI (filled in by Klenow treatment), XbaI *URA3* fragment of *H. polymorpha* (Merckelbach *et al.*, 1993) between the MluI (filled in by Klenow treatment) and SpeI sites of the 2.7-kb *PEX7* fragment in pUC19. The disruption cassette was isolated by digestion with NsiI and NheI, and transformed to NCYC495 *leu1.1 ura3*. Transformants were selected and tested for correct integration using PCR and Southern blot analysis (data not shown).

Disruption of *PEX12*

An *H. polymorpha pex12* strain was isolated in a screen of random integration of linear DNA fragments (RALF) mutants that were unable to grow on methanol (van Dijk *et al.*, 2001). Sequence analysis revealed that, in this mutant, the linear p-REMI fragment was inserted between base pairs 55 and 56 of the *PEX12* gene, and hence represented a *PEX12* disruption mutant. The *PEX12* gene was cloned by functional complementation of the original RALF mutant and deposited at GenBank (accession number AF333026).

Biochemical methods

Crude extracts were prepared as described previously (Baerends *et al.*, 2000). Protein concentrations were determined by the Bradford assay using the Bio-Rad protein assay kit (Bio-Rad GmbH, Munich, Germany), using bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described previously (Laemmli, 1970). Gels were subjected to Western blotting (Kyhse-Andersen, 1984), and nitrocellulose blots were decorated using the chromogenic (NBT-BCIP) (NBT, nitro-blue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3′-indolyphosphate p-toluene salt) or chemiluminescent (peroxidase) Western blotting kit (Roche) and specific polyclonal antisera against various *H. polymorpha* proteins.

Microscopy

Cells were fixed and prepared for electron microscopy and immunocytochemistry as described previously (Waterham *et al.*, 1994). Immunocytochemistry was performed on ultrathin sections of Unicryl-embedded cells, using specific polyclonal antisera against various *H. polymorpha* peroxisomal proteins and gold-conjugated goat anti-rabbit serum (Waterham *et al.*, 1994).

Results

Hansenula polymorpha pex cells contain four distinct groups of peroxisomal structures

We restricted our study to *PEX* genes that play a role in peroxisome biogenesis (i.e. matrix protein import and membrane formation), namely *PEX1–PEX20*, excluding *PEX11*, which is involved in controlling peroxisome numbers (Erdmann & Blobel, 1995; Marshall *et al.*, 1995). *Hansenula polymorpha* Pex1p–Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex17p, Pex19p and Pex20p have been identified before in the laboratory (Veenhuis *et al.*, 2003). The two peroxins Pex9p and Pex15p that also have been implicated in peroxisomal matrix protein import could not be identified in *H. polymorpha* (Kiel *et al.*, 2006). *Hansenula polymorpha* also does not contain the *S. cerevisiae* Pex7p auxiliary proteins Pex18p and Pex21p, but contains Pex20p instead (Purdue *et al.*, 1998; Otzen *et al.*, 2005).

From the group of *PEX* genes that are thought to play a role in peroxisomal membrane formation, *PEX3*, *PEX16* and *PEX19*, *PEX16* has so far only been identified in mammals, plants and the yeast *Yarrowia lipolytica*, but is absent in *H. polymorpha* (Kiel *et al.*, 2006).

Cells of the selected *H. polymorpha PEX* deletion strains were grown in glucose-limited chemostat cultures using choline as sole nitrogen source. Samples were taken from

steady-state cultures for morphologic and biochemical analysis.

Electron microscopy revealed that all *H. polymorpha pex* mutants studied could be placed in four morphologically distinct groups (Fig. 1). The first group is characterized by the presence of almost normal peroxisomes (Figs 1a and 2a). This group includes *H. polymorpha pex7* and *pex20* cells, which are defective in PTS2 (PTS, peroxisomal targeting signal) protein import, as well as *pex17* (Otzen *et al.*, 2005). However, almost normal peroxisomal structures were also observed in *H. polymorpha pex17* cells (Fig. 2a). These organelles were reduced in size relative to those observed in cells of wild-type *H. polymorpha*, but still harbored significant amounts of AO and DHAS proteins (Fig. 2c and d). This was an unexpected observation, as *S. cerevisiae* and *P. pastoris pex17* mutants contain only very small peroxisomal structures or membrane remnants (ghosts) (Snyder *et al.*, 1999; Harper *et al.*, 2002).

The cells in the second group of mutants contained very small remaining peroxisomal structures, which harbored a matrix space. These included *H. polymorpha pex2*, *pex4*, *pex5*, *pex10*, *pex12* and *pex14* (shown for *pex2* in Fig. 1d). Apparently, these structures contain very limited amounts of matrix proteins (Komori *et al.*, 1997).

The third group of *pex* mutants harbors multilamellar membrane sheets that lack an apparent organellar matrix. Members of this group are *pex1*, *pex6*, *pex8* and *pex13* (shown for *pex13* in Fig. 1c). This suggests that matrix protein import is fully blocked in these mutants.

The final group comprised *H. polymorpha pex3* (Fig. 1b) and *pex19* cells, which lacked recognizable peroxisomal membranes.

Peroxin levels in *H. polymorpha pex* mutants

We subsequently analyzed the various *pex* mutants for peroxin contents. Crude extracts, prepared from chemostat-grown

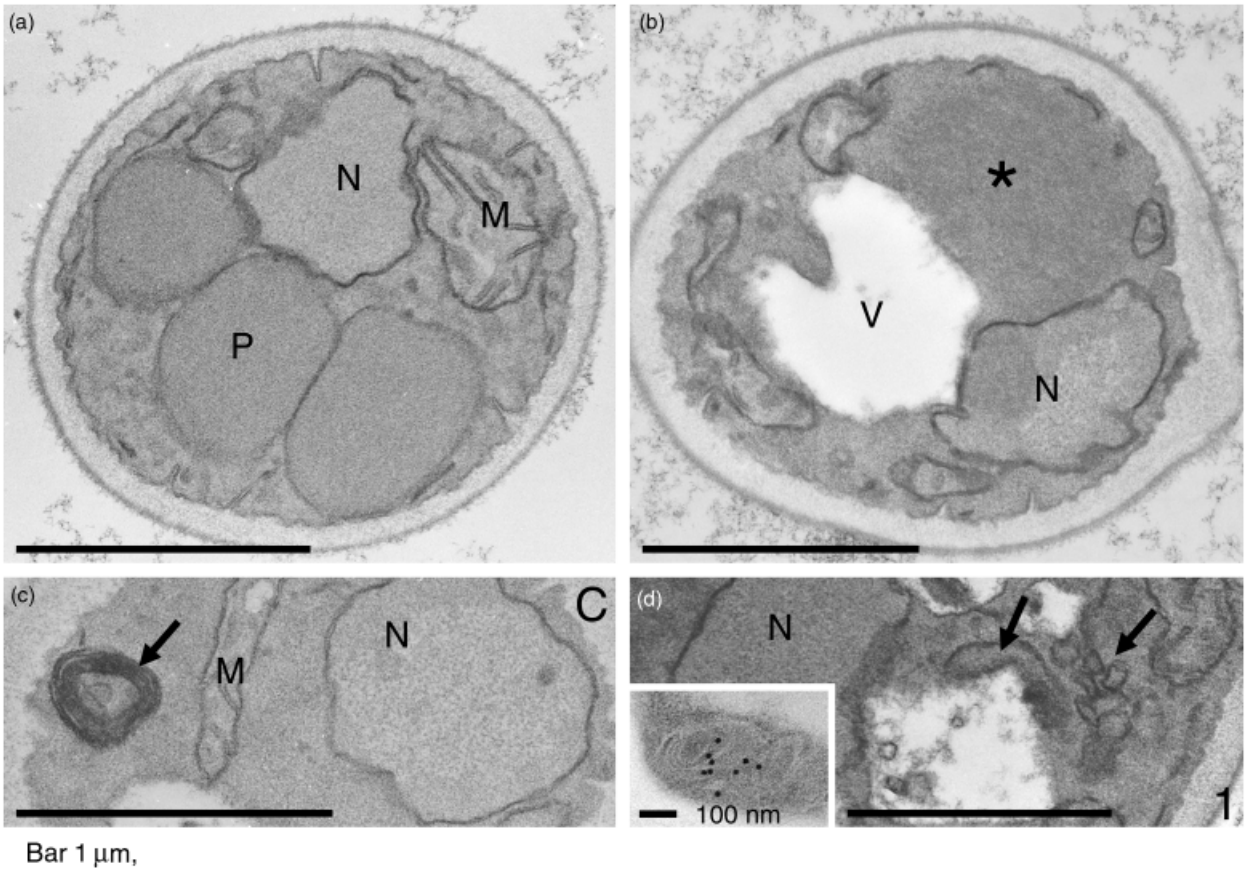

Fig. 1. Typical examples of the four morphologic classes of *Hansenula polymorpha pex* mutants that were observed. Ultrathin sections of *Hansenula polymorpha* wild-type, *pex3*, *pex13* and *pex2* cells grown in glucose-limited chemostat cultures at steady state. In wild-type cells (a), three to five peroxisomes are typically present per cell section, whereas in *pex3* cells (b), peroxisomes are completely absent but a cytosolic AO inclusion (*) could be observed. In *pex13* cells (c), only multilayered peroxisomal membrane structures (arrow) were present, and in *pex2* cells (d), numerous small peroxisomal vesicles were observed (arrows). The PTS1 receptor Pex5p is localized to these vesicles, as shown by the immunolabeling with Pex5p antibodies (d, insert). The electron micrographs are from $KMnO_4$-fixed (a–d) or glutaraldehyde-fixed (d, insert) cells. M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. (a, b) Bar represents 1 μm. (c, d) Bar represents 0.5 μm, unless otherwise stated.

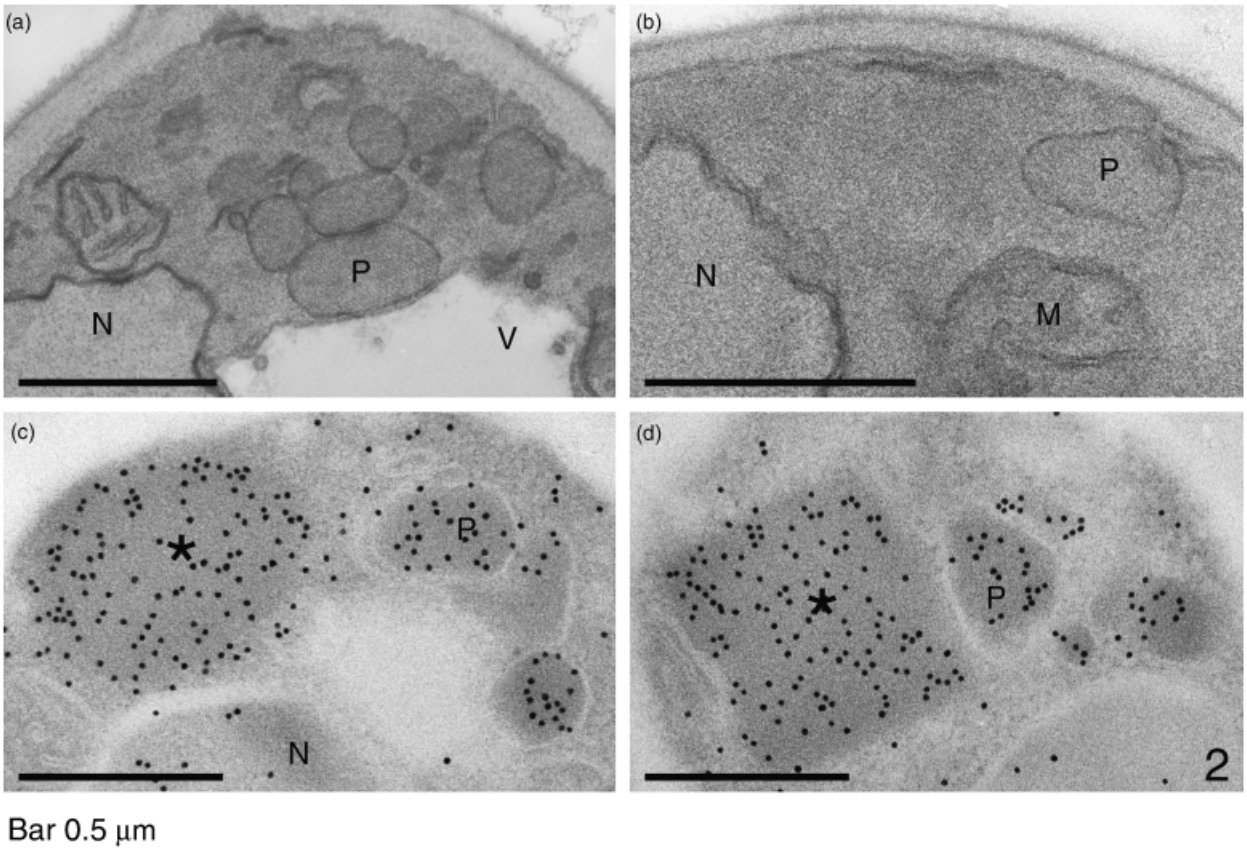

Fig. 2. *Hansenula polymorpha pex17* form peroxisomal structures that harbor matrix proteins. Ultrathin sections of *Hansenula polymorpha pex17* cells grown at steady-state levels in chemostat culture (a, c, d) on glucose/choline, or in batch culture on methanol (b). In chemostat-grown *pex17* cells, several peroxisomes are observed, whereas in batch culture, one small peroxisome could typically be observed. Immunocytochemistry revealed that the organelles contain AO (c) and DHAS (d) protein, and that these proteins are also present in the cytosol, where they are frequently organized in crystalloids (∗). (a, b) $KMnO_4$-fixed cells, and (c, d) glutaraldehyde-fixed cells. P, peroxisome; V, vacuole; N, nucleus; M, mitochondrion. The bar represents 0.5 μm. (b) is from Kuravi VK, PhD Thesis, University of Groningen, 2007.

cells, were analyzed for levels of Pex3p, Pex10p and Pex14p by Western blotting. In addition, we studied the levels of the peroxisomal matrix proteins AO, CAT, DHAS, and amine oxidase (AMO).

The levels of these major matrix proteins were comparable in all *pex* mutants analyzed (shown for CAT in Fig. 3; AMO, AO and DHAS not shown), indicating that the defects in peroxisome biogenesis do not significantly affect the levels of these proteins. This is in line with earlier observations that these enzymes are normally assembled and stable in the cytosol of *pex* mutants (van der Klei *et al.*, 1998).

Except for Pex3p in *pex3* cells, Pex10p in *pex10* cells, and Pex14p in *pex14* cells, these three membrane-bound peroxins were detected in cells of all strains (Fig. 3a and c). Variations in protein levels were observed for Pex14p, but not for Pex3p and Pex10p. We did not observe any direct relationship between enhanced Pex14p levels and *pex* cells that contained matrix space. Moreover, normal levels of Pex3p have been observed before in *H. polymorpha pex19* cells that lack peroxisomal membranes (Otzen *et al.*, 2004).

Discussion

This article describes the characterization of peroxisomal structures that remain in *H. polymorpha pex* mutants and their classification into four distinct groups. Among the *pex* mutant cells that contained normal peroxisomal structures, *pex7*, *pex17* and *pex20*, the presence of *pex17* is remarkable (Fig. 2). *Pex7* and *pex20* were expected to be in this group, as they are affected in PTS2 protein import only. Hence, in these cells, import of the major proteins of methanol metabolism, AO, DHAS and CAT, is not abolished, resulting in the formation of almost normal peroxisomes. However, Pex17p is proposed to represent a component of the PTS1/PTS2 receptor docking machinery. The fact that, in *pex17*, significant amounts of AO, DHAS and CAT are imported

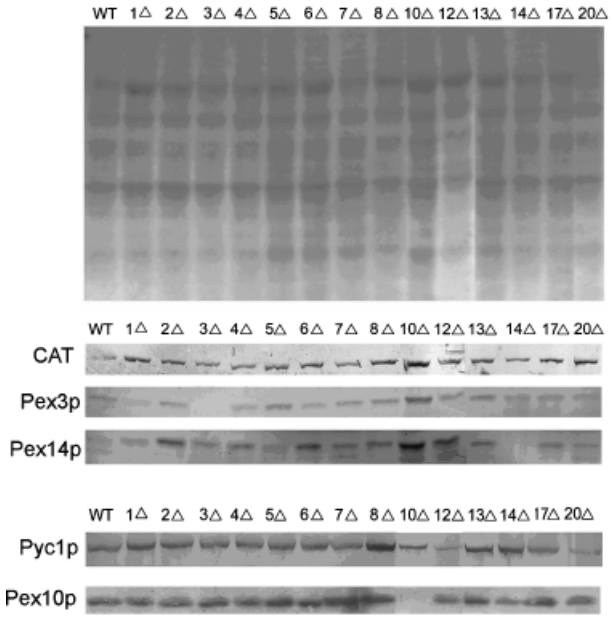

Fig. 3. Matrix protein and peroxin levels in various *pex* mutants. The Western blots (middle and lower panel) show steady-state levels of peroxins in the wild type (WT) and the indicated *pex* mutants, grown in a chemostat on glucose/choline. Equal amounts of protein were loaded per lane, separated by SDS-PAGE, and immunoblotted using antibodies against the indicated *Hansenula polymorpha* proteins. The total protein staining (Ponceau S; upper panel) and a Western blot using antibodies against pyruvate carboxylase (Pyc1p) were included as loading controls. Blots were decorated using antibodies raised against Pyc1p, CAT, Pex3p, Pex10p and Pex14p antibodies.

suggests that Pex17p does not have a crucial function in import. In relation to this, it is also remarkable that deletion strains of the three components of the docking complex, Pex13p, Pex14p and Pex17p, display dissimilar peroxisome phenotypes. The finding that multilaminar membrane structures that lack luminal space were observed only in *pex13* cells (Fig. 1) suggests that this protein is the most important component of the docking complex.

Snyder *et al.* (1999) failed to detect peroxisomes in *P. pastoris pex17* cells. Instead, very small vesicular and tubular peroxisomal structures were observed. Later studies (Harper *et al.*, 2002) indicated that *P. pastoris pex17* cells do contain peroxisomal structures, similar to the few peroxisome-like structures observed in batch-cultured, methanol-induced *H. polymorpha pex17* cells (Kuravi, 2006). In our chemostat cultures, however, peroxisomes of normal size were observed. These observations illustrate that the rate of peroxisome formation in yeast *pex* mutants is directly related to the experimental (e.g. cultivation) conditions. Hence, it cannot be ruled out that other yeast *pex17* species, in which, so far, no structures reminiscent of peroxisomes have been detected (Huhse *et al.*, 1998), may also be able to form peroxisomal structures under different growth conditions. These findings stress the importance of using controlled cultivation schemes when analyzing yeast *pex* mutants in comparative studies (e.g. in genomics, transcriptomics, and proteomics).

Hansenula polymorpha pex2, *pex10*, *pex12* and *pex4* belong to the same morphologic class as *pex14* (Fig. 1). The luminal space observed in *H. polymorpha pex4* and *pex14* cells is in line with previous observations that these structures contain limited amounts of matrix proteins (Purdue *et al.*, 1998; van der Klei *et al.*, 1998).

Pex2p, Pex10p and Pex12p are ring finger proteins that have been implicated in peroxisomal matrix protein import. The presence of a luminal space in these mutants suggests that some residual peroxisomal matrix protein import is possible in the absence of either one of these ring finger proteins. Hence, in these mutants, the defect in the import process most likely occurs after docking and translocation, as in *pex4* and *pex14* mutants. Possibly, in these mutants, Pex5p is defective in that it cannot reach the receptor recycling machinery. The latter suggestion is based on previous findings that *H. polymorpha* Pex5p accumulates at peroxisomal ghosts in *pex2*, *pex10* and *pex12* cells (Fig. 1d). Also, in human cell lines obtained from patients suffering from a peroxisome biogenesis disorder due to a mutation in *PEX10* (complementation group 7) (Warren *et al.*, 1998), HsPex5p was shown to accumulate inside peroxisomes (Dodt & Gould, 1996).

The last morphologic group of *H. polymorpha pex* strains includes *pex1*, *pex6*, *pex8* and *pex13*, which have ghosts lacking a distinct lumen (Fig. 1). These findings suggest that import of matrix proteins in these mutants is strongly affected. This is in agreement with the proposed important function of Pex13p in receptor docking. The intraperoxisomal peroxin Pex8p plays a key role in peroxisomal matrix protein import in *H. polymorpha* (Waterham *et al.*, 1994), where it is proposed to mediate dissociation of the PTS1 cargo from Pex5p (Wang *et al.*, 2003), but is also important for associating the two core complexes for matrix protein import [the Pex13p–Pex14p–Pex17p docking complex and the Pex2p–Pex10p–Pex12p ring finger complex (Agne *et al.*, 2003)]. The latter most likely explains why, in the absence of Pex8p, peroxisomal matrix protein import is severely blocked.

Pex1p and Pex6p are AAA-ATPases (AAA, ATPases associated with various cellular activities) that form a complex at the peroxisomal membrane (Kiel *et al.*, 1999). Data from epistasis analysis indicated that *P. pastoris* Pex1p and Pex6p function at a very late stage of the Pex5p import cycle (Collins *et al.*, 2000). This possibility was recently experimentally tested and confirmed (Platta *et al.*, 2004). Also, Pex4p is important at a very late stage of the matrix protein import process. Interestingly, the morphologic peroxisome phenotype of *H. polymorpha pex1* and *pex6* cells differs from

that of *pex4* cells, which could be explained by the fact that these peroxins are not equally important in receptor recycling. Alternatively, the *pex1* and *pex6* phenotypes may also be due to additional functions of Pex1p and Pex6p, e.g. in the formation of peroxisomes from the endoplasmic reticulum (ER). Studies in *Y. lipolytica* revealed that Pex1p and Pex6p are important for the formation and fusion of ER-derived vesicles, processes that are required to form new peroxisomes from the ER (Titorenko & Rachubinski, 2000). Hence, the peroxisomal membrane remnants that were detected by electron microscopy in *H. polymorpha pex1* and *pex6* cells may also include early intermediates of peroxisome formation from the ER.

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